Downstream Processing
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Lecture - 28
Chromatography (Continued)

We shall continue with the topic of chromatography. We were talking about the ion exchange chromatography that is the chromatography used, when you want to separate ionic metabolites or ionic proteins. It could be an ion ionic it could be cationic, or it could be both together.

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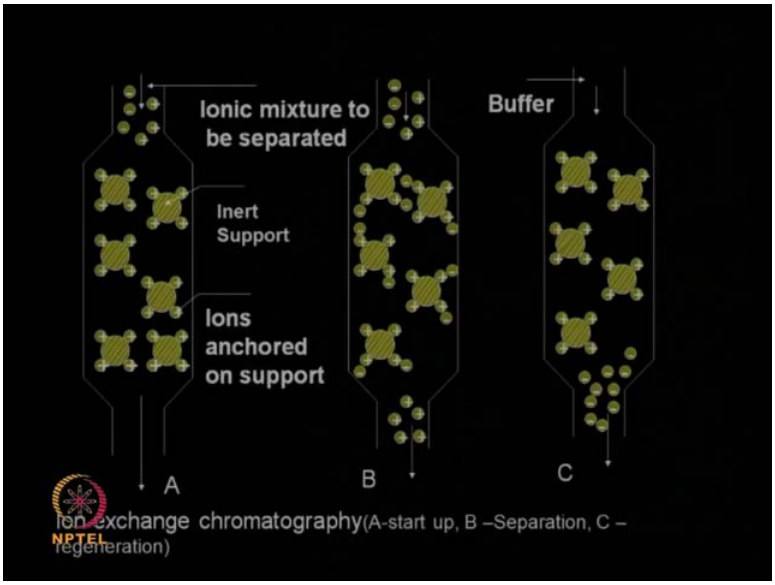

So, what happens in ion exchange chromatography. We have a inert support, now a days we they use more of a polymeric base support, I you have ions. See in this particular example, we are showing positive ions all anchored on to the inert support. Now, suppose you are introducing a mixture of cations and anions, what will happen? The negative ions will bound to the positive ions. So, there exhaust stream will have only positive ions coming out.

Once, you have done the suppression by you can change the buffer conditions, like you can change the salt concentration or you can change the pH. So, that whatever negative ions that are bound will come out. So, this is one way of separating positive and negative

ions, suppose you have both cations and anions anchored to the support. Then we can remove the entire salt from a mixture.

For example, I am using a salt for salting out of a protein such a solution will have proteins and salts. And ion exchange chromatography containing both cations, and anions could be one way of removing the salts first, and then you can go into purifying the protein of interest. So, ion exchange chromatography is based on the concept of the ions suppression of ions either it could be ions of interest, which could be concentrated or ions of not interest could be captured. So, both can be adopted.

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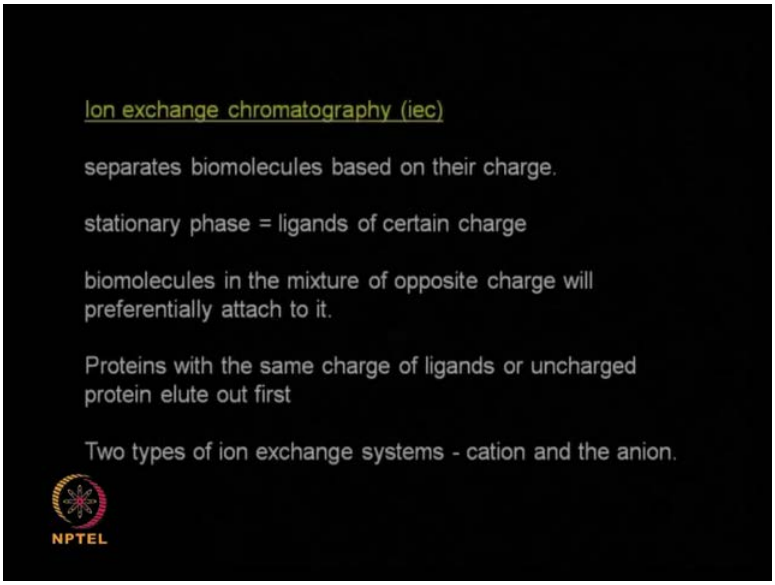

So, the stationary face will have ligands of certain charge. So, the bio-molecules in the mixture of opposite charge will preferentially, attach to it. And proteins or molecules with the same charge will be eluting out, even uncharged proteins will be eluting out first. Now, you have two types of ion exchange chromatography. That means, cation as well as anion or you can have mixtures of both cation and anionic ligands present inside the chromatography.

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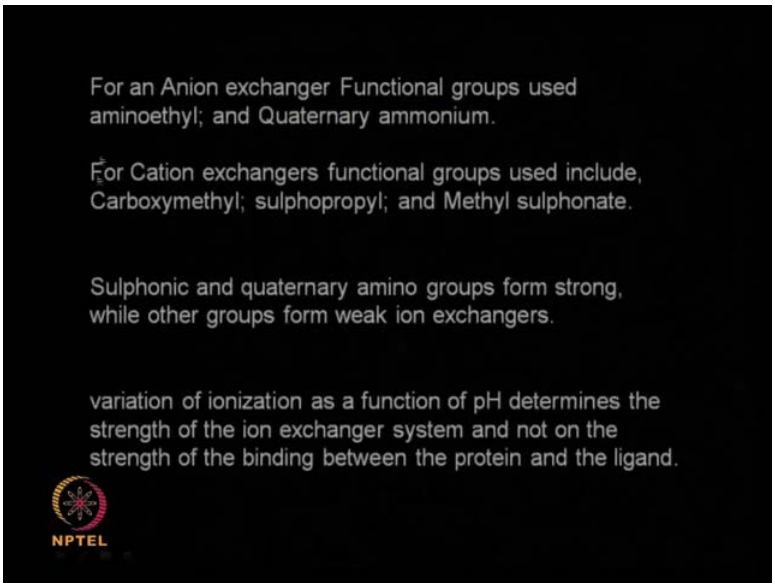

So, for example if you looking at an anion exchanger, the functional groups used could be aminoethyl or quaternary ammonium, so there will be a plus charge on the nitrogen. For cation exchangers functional groups like carboxymethyl, sulphopropyl, methyl sulphonate and so on actually. Sulphonic and quaternary amino groups form strong while other groups form weak ion exchangers.

So, if you are varying the ionization as a function of pH, it determines the strength of the ion exchanger it does not tell you anything about the strength of the binding protein and the ligand. It just tells about the strength of the ionic changer system.

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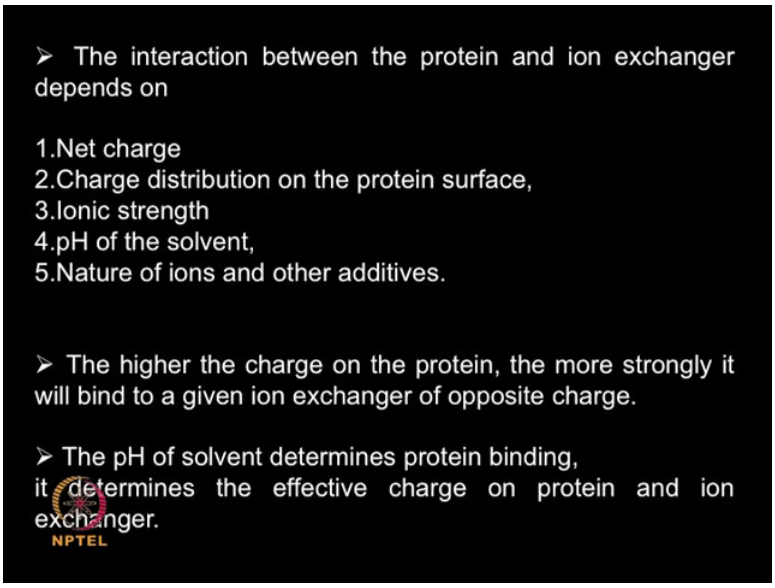

So, the interaction between protein and the ion exchanger depends on the net charge. So, higher is the charge higher will be the binding capacity, charge distribution on the protein surface. How the positive and negative charges are distributed on the protein surface, ionic strength pH of the solvent, nature of ions that are present and presence of other additives there are present in side.

Higher the charge on the protein, more strongly it will bind to a given ion exchange of opposite charge obvious, right? Also the pH of the solvent determines the protein binding it also determines, the effective charge on the on the protein and ion exchanger. So, if the protein is very high charge it will bind strongly to the ion exchanger system and if the pH of the solvent is different in the protein binding capacity also becomes different.

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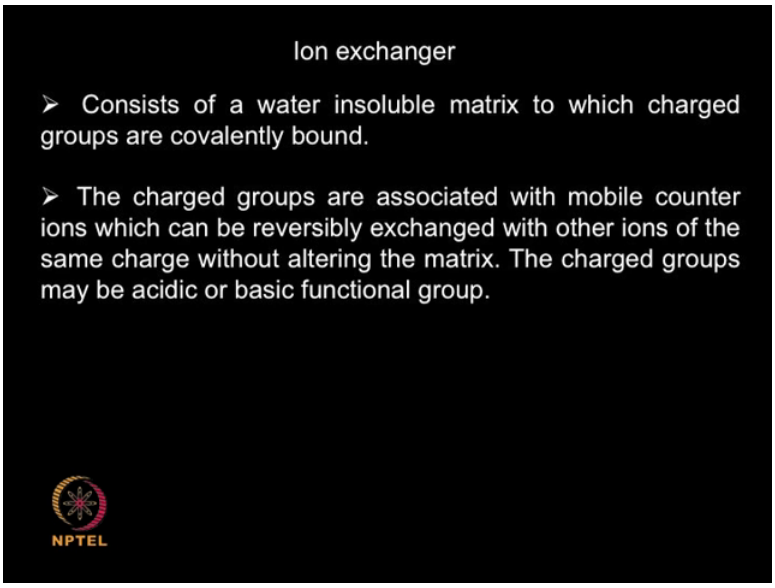

So, what does an ion exchanger contain? It consists of a matrix that is an insoluble matrix that will be the base on which, the ionic ligand are immobilized pre-equivalent bound. The charge groups could be acidic, or charge groups could be basic functional groups.

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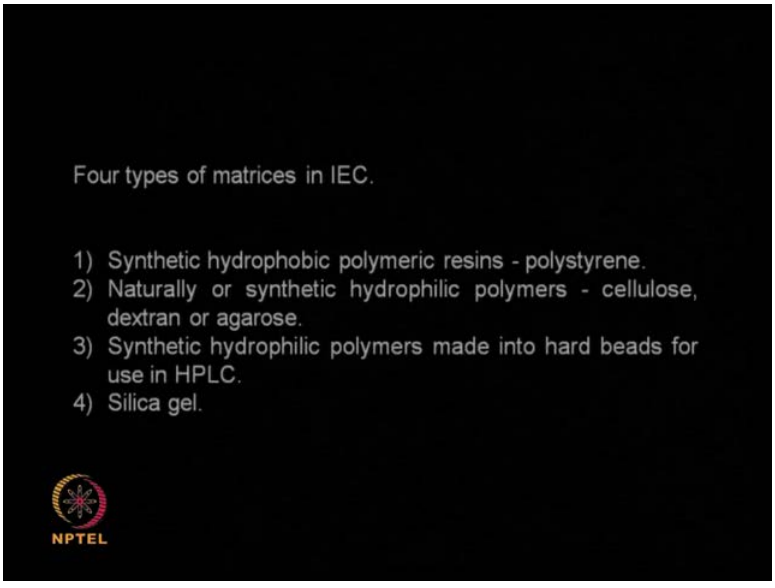

So, we can have four types of matrices you can have synthetic hydrophobic polymeric resins, like polystyrene. Polystyrene is pretty hydrophobic natural or synthetic hydrophilic polymer. So, when you see natural it could be cellulose, dextran, agarose and

so on. It could be synthetic hydrophilic polymers made into hard beads use in HPLC system. So, what is the advantage? We can use vigorous method for washing and cleaning recycling reusing, so that is an advantage. What we can use silica gel based method? So, you can have different types of matrices in an ionic change column.

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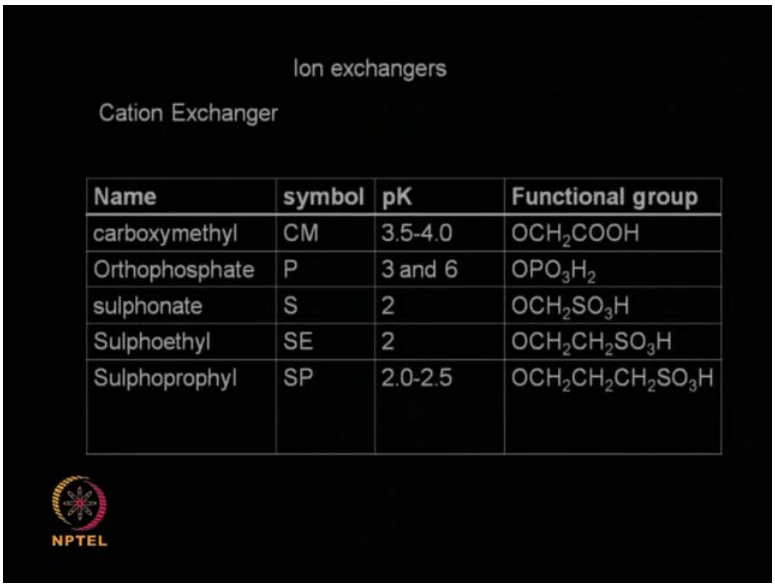

So, a typical ion exchangers if you have cationic exchanger, I could use carboxymethyl, we can use orthophosphate, we can use sulphonate, we can use sulphoethyl, sulphoprophyl and so on. So, each one has different pK value, as you can see here. So, depending upon the ions you would like to separate, then you can use of different types of cationic ligands anchored to the stationary faces.

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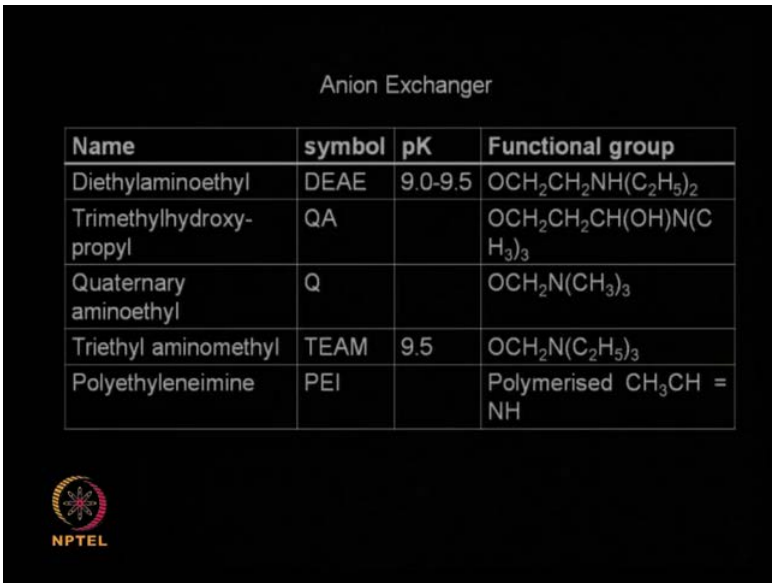

Suppose, we are looking at anionic we can use diethylaminoethyl, trimethylhydroxypropyl, quaternary aminoethyl, triethyl aminomethyl, polyethyleneimine. So, you see lot of nitrogen containing groups are here, most of this. So, that is how you get the charge N plus charge. Whereas, in the previous case we have lot of O H type of groups, which gives you a negative charge. Again, if you look at the pK values they are more in the basic side, whereas in the other case they are more in the acidic side.

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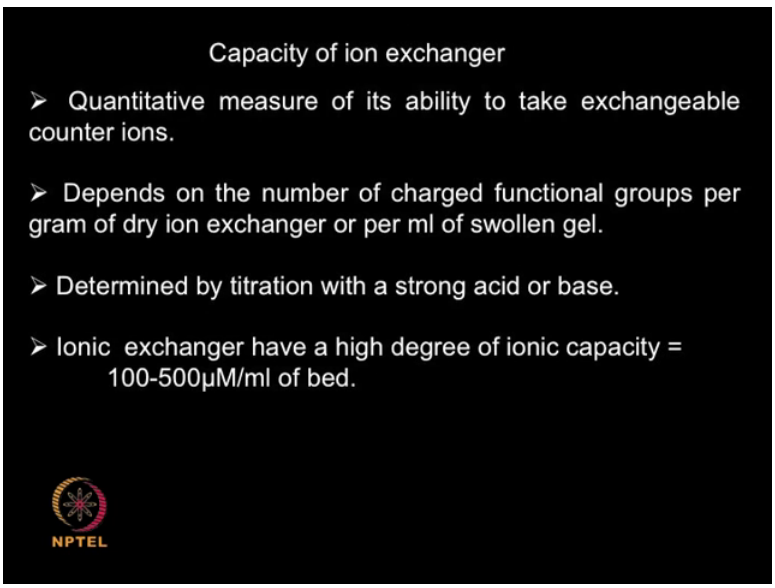

So, what determines the capacity of ion exchanger? Capacity is the quantitative measure of the ability of the ion exchange system to take in the counter ions. So, it depends on the number of charges functional groups present inside, a column per gram dry ion exchanger per ml of swollen gel.

So, how do you determinate? So, we can titrate it with strong acid or base and then we can find out how much of the acid, or base required to neutralize the ion exchange system. So, you can have say like a 100 to 500 micro molar per ml of beads, beads you know beads of bed. So, that is very high degree of ionic capacity.

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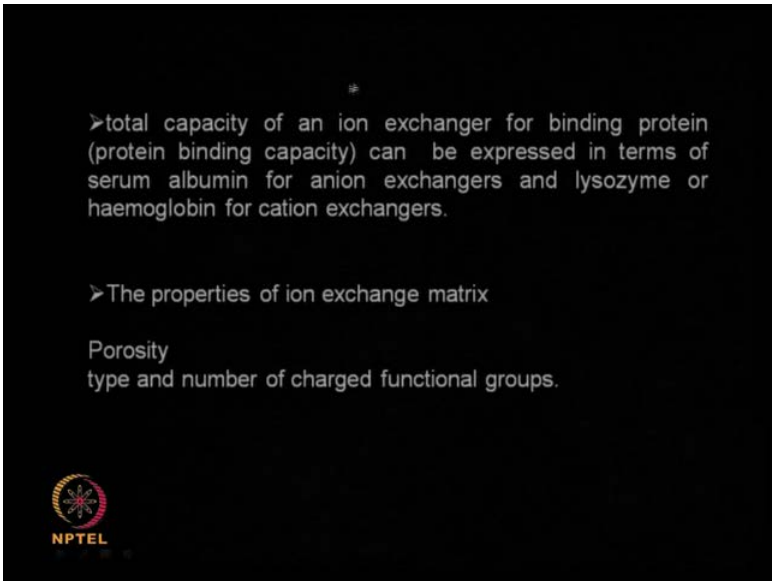

So, the total capacity of an ion exchanger for binding protein can be expressed in terms of serum albumin for anion, or lysozyme, or haemoglobin for cation exchangers. So, generally the capacity is based on, if it is an anion the amount of serum albumin it can be exchanged if you change cation, the amount of lysozyme or haemoglobin that can be exchanged.

So, the property of ion exchange will depend on porosity as well, because highly pores material we can have more charge ligand bounds to it. Type of charges functional groups present on the stationary matrix, number of charge functional groups present on the stationary matrix. So, all these properties decide on the ion exchange matrix.

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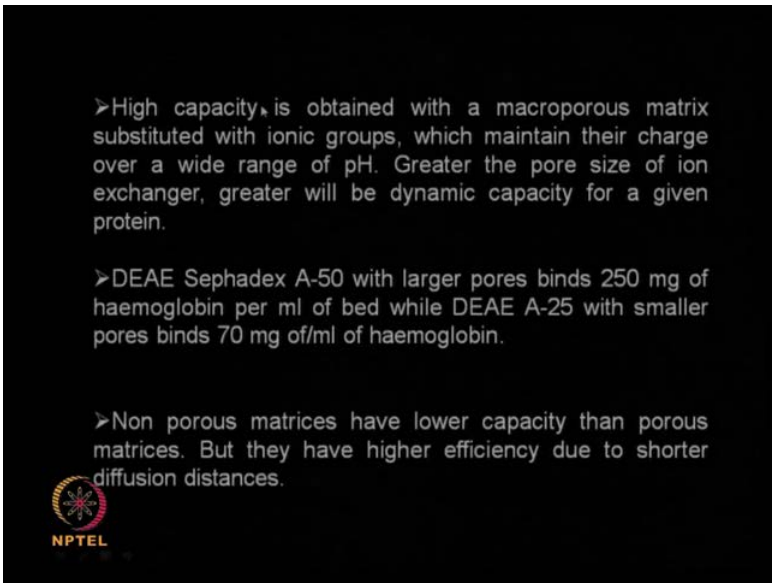

So, how do you get high capacity in an ion exchanger matrix? We can have highly macro porous matrix, we can substitute the ionic groups, which maintain their charge even over a wide range of pH that is also very important. So, greater the pore size of an exchanger, greater will be the dynamic capacity for a given protein. For example, if you take the DEAE sephadex A-50, which as large pores binds about 250 milligram of hemoglobin per ml of bed that is a bit dumber 250 milligrams of hemoglobin per ml bed.

Whereas, if you have A-25 with smaller pore it binds only 70 milligrams of hemoglobin per ml. So, we can achieve 250 milligrams of hemoglobin per ml or we can achieve 70 milligrams of hemoglobin per ml depending upon the pore distribution, and pore size. Non pores matrix will have lower capacity then pores matrix that is obvious, but non pores matrix will higher efficiency, because they molecules do not have to defuse to the pores. So, the pore diffusion is 0, so it is having less diffusion distances. So, capacity will be higher.

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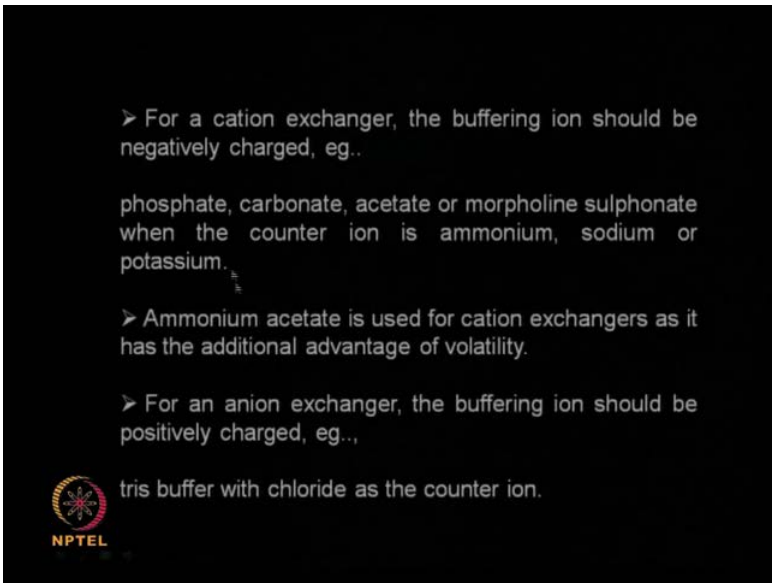

For a cation exchanger, the buffering ion should be negatively charged like phosphate, carbonate, acetate or morpholine sulphonate. When the counter ion is ammonium, sodium, or potassium. So, ammonium acetate is used for cation exchangers, because it has the additional advantages volatility. Whereas, for an anion exchanger the buffering ion should be positively charged, like tris buffer with chloride as the counter ion.

So, if you have a cation exchanger, the buffering ion should be negatively charged. If you have an anionic exchanger the buffering ion should be positively charged. So, for a cation exchanger the buffering ion should be phosphate, carbonate, acetate, morpholine, sulfate and the counter ion is the ammonium, sodium or potassium correct. Whereas, if it is anion exchanger, we could use tris buffer with chloride as the counter ion because for an anion exchanger we need a positively charged buffering ion.

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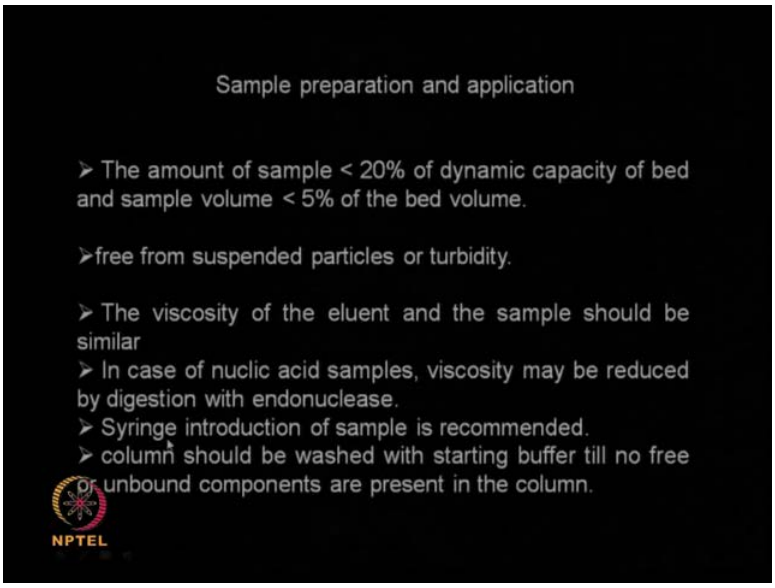

So, how do you go about preparing the sample, for an ion exchanger we need to prepare the sample. So, the amount of sample should be 20 percent of demonic capacity of the bed, and sample volume should be less than 5 percent of the bed volume. So, we do not want to over load the bed, then becomes very, very inefficient. The sample should be free from suspended particles, or turbidity because we do not want turbid material binding to the ions.

Viscosity of the eluent and the sample should be similar, if the eluent and that is the continuous face and the sample are different then you are going to have different with the flow.

So, in case of nucleic acid sample viscosity may be reduced by digestion with the endonuclease. Syringe introduction of sample is always is recommended. Column should be washed, which starting buffer till no free or unbound components are present in the column. So, we have to keep washing, washing and till whatever is bound gets totally removed, the column is free from any ions bounds to the ion exchanger. So, even after each analysis or after each suppression we need to wash the column thoroughly using a buffer solution.

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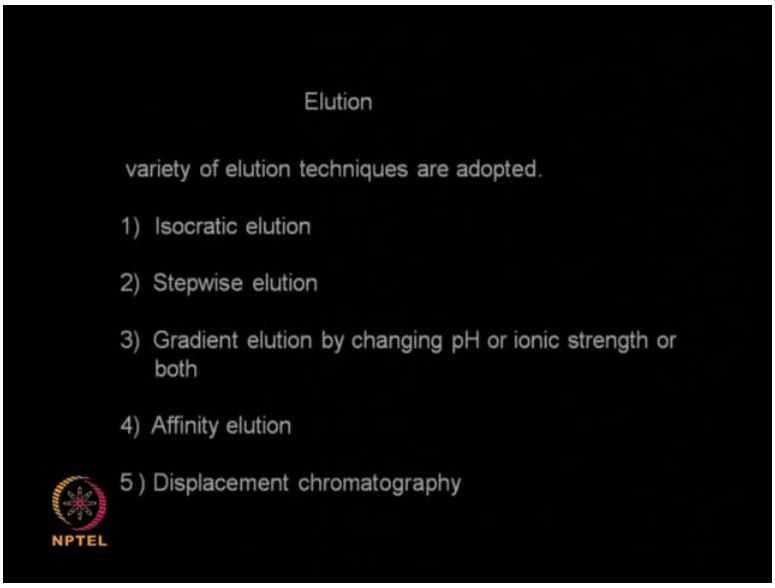

So, variety of valuation techniques can be used, we can use and isocratic elution that means, we use single solvent, single strength, continuously flowing throughout the suppression process. We can use step vise elution that means, we can slowly change the strength of the salt in terms of steps. We can have a gradient elution by changing the pH or ionic strength or both.

That means, we can slowly linearly change the pH over a period of time, we can affinity elution that means, we can add certain affinity ligands. We can have displacement chromatography that means, we can have another yes, buffer solution which will displace whatever has been bound to the ion exchange column. So, we can think of different elution techniques for suppression, as well as for later activation of the column, as well as removal of ions which are bound to the stationary faces.

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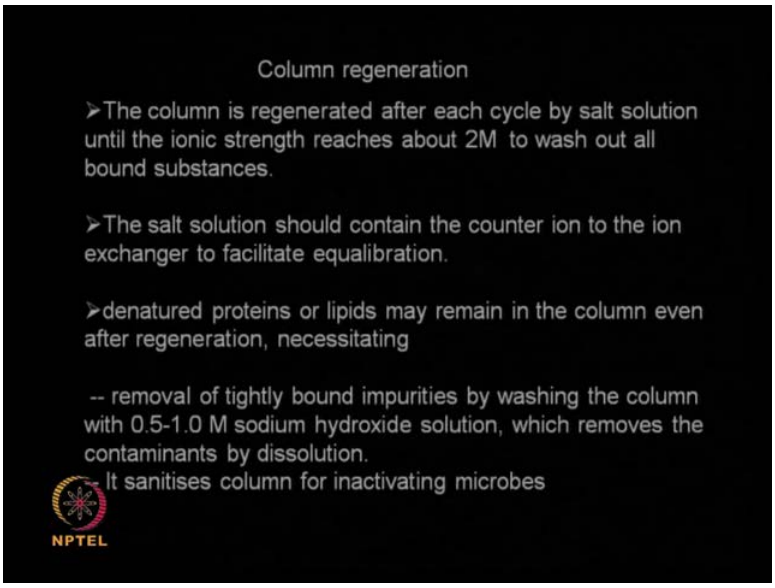

So, the column regeneration is a very, very important aspect after you have done the ion exchanger suppression process. Like I mentioned that we need to keep on washing with buffer until whatever is bound gets removed. So, like a salt solution. So, we can introduction salt solution, until the ionic strength reaches about 2 molar to wash out what ever has been bound to the ionic exchange stationary matrix. So, the salt solution should contain counter ion to the ion exchanger to facilitate equilibration that is very, very important actually.

So, there should be the counter ion, which will remove the ions that are bound to the ion exchange matrix. Sometimes denatured proteins or lipids may remain in the column even after regeneration. There may be some tightly bound impurities. So, what do we do? Then we have to removal of tightly bound impurities, or denature proteins by washing the column with 0.5 to 1 molar sodium hydroxide solution know.

So, we have to resort to very harsh condition like using sodium hydroxide. So, it will remove all the contaminants number one, number two it will also inactivate in there are any microbes present. So, that way we are something like we are sterilizing our system. So, the microbes don’t group and forms bio-film inside your ion exchange system.

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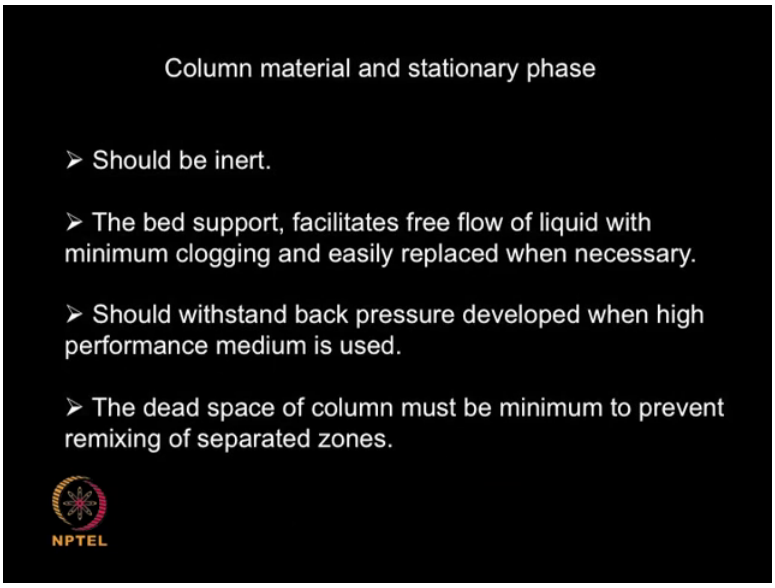

So, what are the column material or the stationary face we need to consider? The column material should be inert, the bed support should facilities free flow of liquid with minimum clogging that's very, very important if you going to have a clogging. You are going to create pressure inside the column and you are creating a back pressure. So, the pump, which is pumping the solution inside is going to have problem. And the column material should be easily replaceable and then we, so that we can charge with fresh material inside.

It should be stand back pressure developed when high performance medium is used. That means, you should not crumble when we apply very high pressures. The bed space inside the column after a pack should also be a minimum, because if you have dead space we are going to improper mixing.

So, we will have zones of good mixing and good suppression we will have zones of improper mixing and bad suppressions. So, the uniformity of suppression is going to very, very poor. So, there should not be dead space inside your back column. So, these are certain requirements for the column material and hence, we need to design column materials so, that all these conditions are satisfied.

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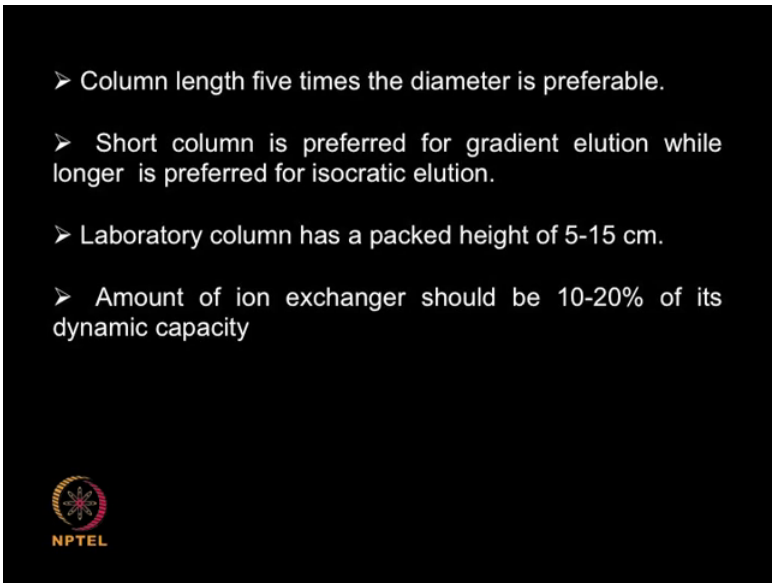

The column length should be 5 times the diameter of the column. Short column is preferred for gradient elution while, longer is preferred for isocratic elution. So, if you are thinking about only isocratic that means, using only one solvent of certain strength through the chromatography process, then it is better to have very long column actually. So, generally if you look at laboratory column they will have a pact height varying between 5 to 15 centimeters, 15 centimeters almost of food. So, it is not very, very long actually and the amount of ion exchanger should be 10 to 20 percent of its dynamic capacity.

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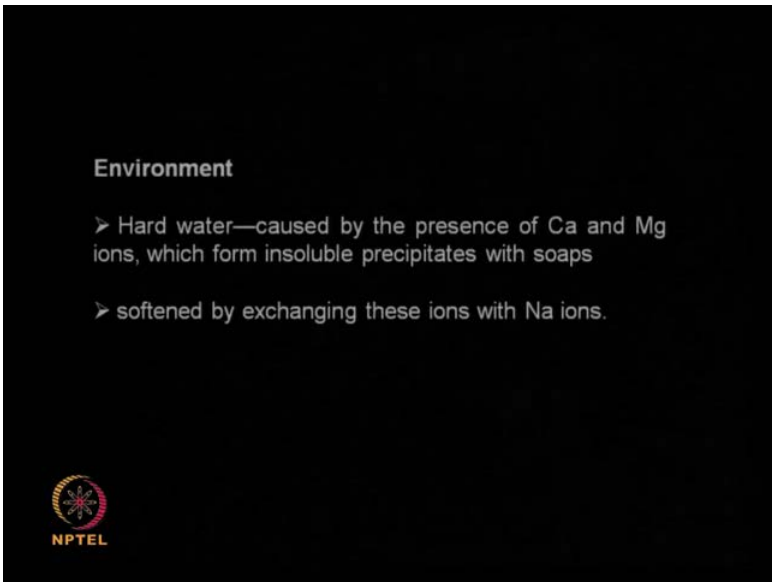

Anion exchange systems are used quit a lot in a hard water purification especially, for purification of well water or river water, which contains too much hardness. Hardness is cast by the presence calcium and magnesium ions. So, it forms in soluble precipitate just like, so actually. So, we can soften the water or we can soften the well or deep bore well water using an ion exchange system. We can use sodium ions so, that the calcium and magnesium all replaced with sodium ions. So, ion exchange systems are the best if you want to soften water.

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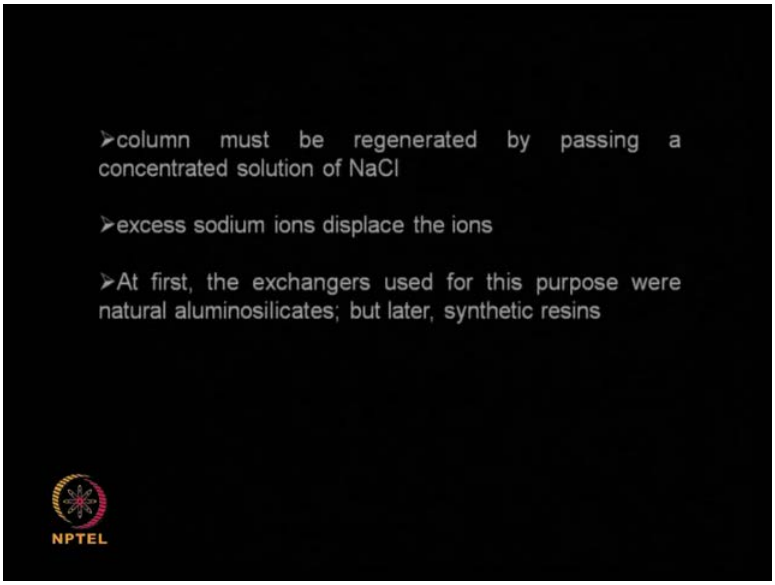

The column was be regenerated of course, by passing concentrated solution of sodium chloride. So, we cannot keep on using the same column for processing large amount of hard water after sometime the calcium and magnesium's, ions will completely saturate the ion exchanger. So, we need to pass sodium chloride. So, what will happen? Excess sodium ions will displaces the calcium and magnesium ions that are bounds to the stationary matrix.

So, originally this type of stationary faces over made up of natural aluminosilicate, but then long term stability of these inorganic material, where not as good as synthetic polymeric resins because synthetic polymeric resins, can survive much harsher conditions, can survive much longer duration of operation, and also several cycles of operations.

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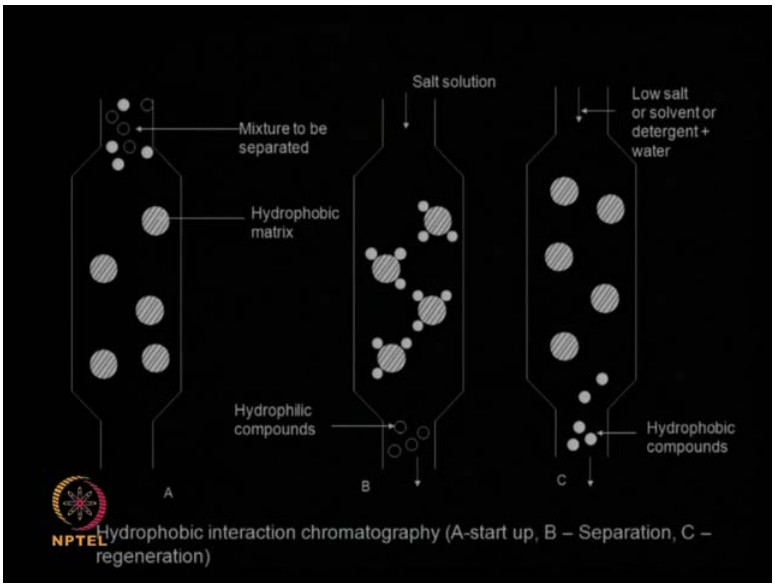

Now, having talked about having ion exchange let us move to the next type of possible suppression that is based on the deference in the hydrophobicity. So, proteins have hydrophobic and hydrophilic groups present on the top. So, sometimes the hydrophobic groups may be embedded inside, then such groups will not be able to contribute in the suppression process, but sometimes these hydrophobic groups may be present outside, because of the folding of the protein.

So, generally hydrophobic groups are inside because proteins are always present in aqueous environment, which is hydrophilic. So, hydrophobic groups are always present inside, but at some time because of the folding constrains these hydrophobic groups are present outside.

So, proteins may have different patches of hydrophobicity and this particular property is made use of in the area of hydrophobic interaction chromatography. So, how does it happen in this particular chromatography? We have the matrix with hydrophobic groups. So, suppose you have a mixture of proteins with hydrophobic and hydrophilic nature, then what will happen all the hydrophobic proteins will bind to the hydrophobic matrix, the hydrophilic proteins will be a eluted out first. Later on we can change the buffer, or we can add a detergent or we can add a salt or we can add solvent and so on.

So, what are that is bound the hydrophobic proteins bound get released and they come out. So, initially we will have predominately hydrophilic protein and later on, during the

regeneration we will have hydrophobic protein. So, this is a good way of separating these 2 type of proteins. So, this is based on the hydrophobic nature of the on the surface of the protein. So, more hydrophobic it is more bound it is going to be, less hydrophobic it is relatively it is going to come out first.

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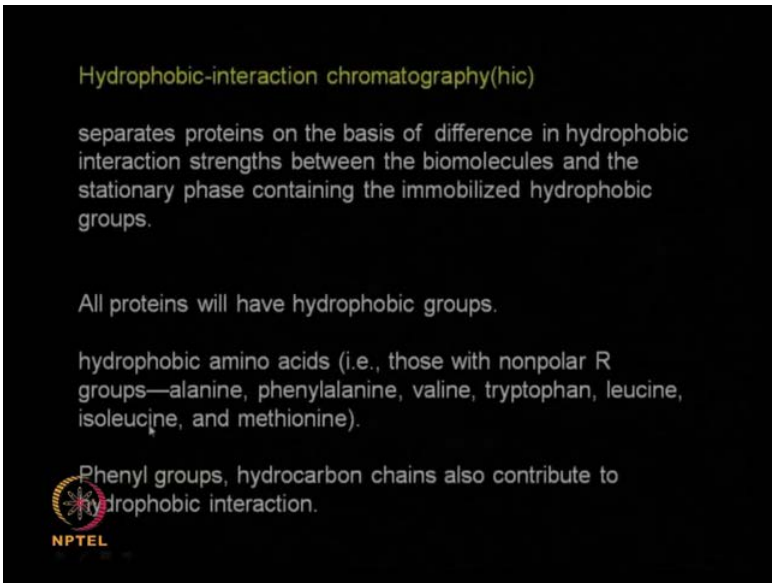

So, all proteins will have hydrophobic groups, but the problem is mostly these hydrophobic groups are embedded inside. So, it is like hydrophobic amino acids that is those with non polar r-groups like alanine, phenylalanine, valine, tryptophan, leucine isoleucine, methonine and so on.

Even final groups hydrocarbon chains they all contribute towards the hydrophobic interaction part a bed actually. So, lot of these groups lead to the hydrophobic nature of the protein.

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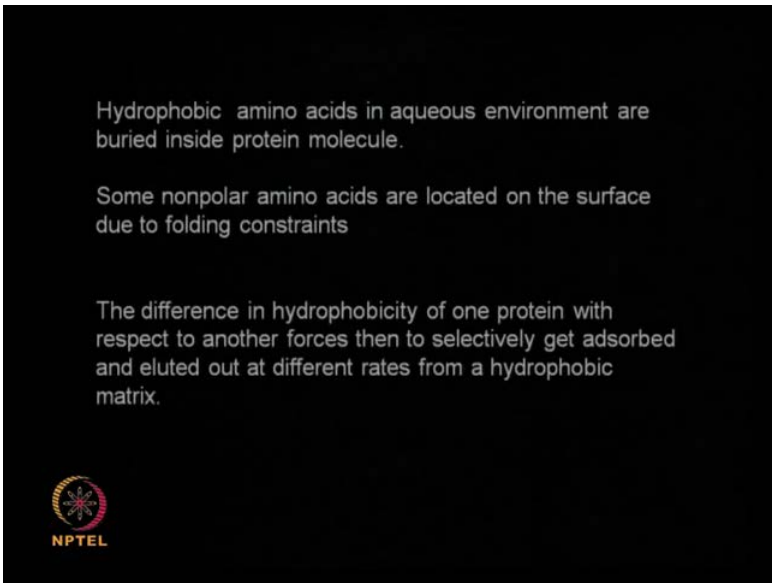

So, generally as I said in an aqueous environment, these are buried inside the protein, but sometimes non polar amino acids are located outside due to the folding constrains. So, if you have proteins deferent hydrophobic surfaces, then you can separate out using the hydrophobic interaction chromatography.

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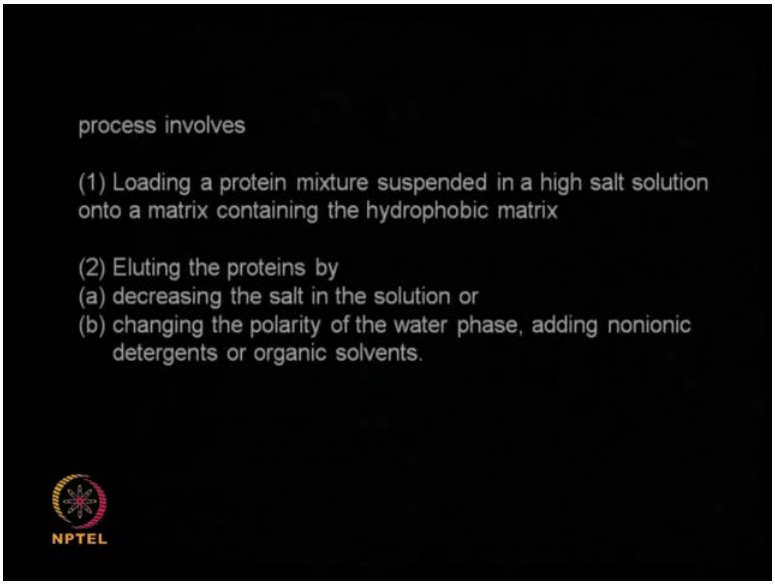

So, what is the process involved? It involves loading a protein mixture suspended in a high salt solution on to a matrix containing hydrophobic matrix, eluting the protein by decreasing the salt in the solution. So, what are you doing you are reducing the salt. So,

protein will go more into the solution, and they get bound to the surfaces depending upon the extant of hydrophobicity.

Later on we will change the polarity of the phase by adding nonionic detergents, or organic solvent during the process, what are the hydrophobic proteins that I have been attached to the hydrophobic matrix matrices will get released. So, these are the various steps in the entire process of hydrophobic interaction chromatography.

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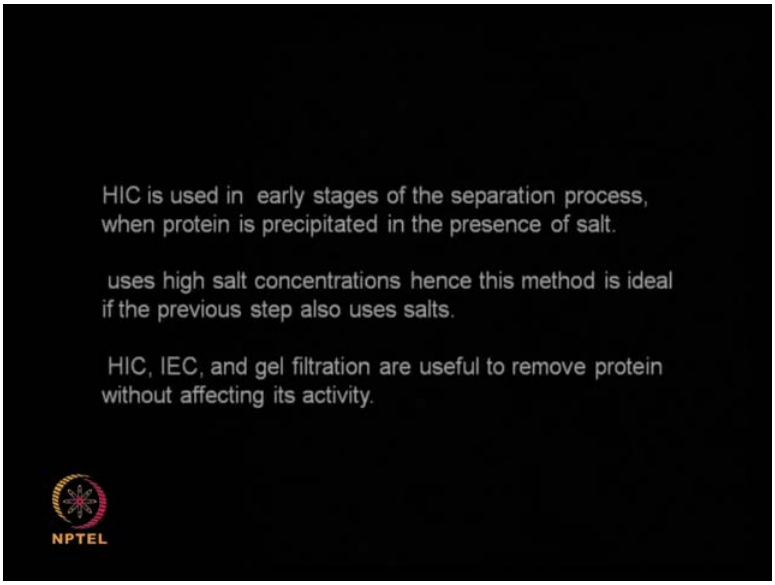

So, it can be used in the early stage of the suppression process especially, when the protein is precipitate in the presence of salts. And also it uses high salt concentration, we are using very high salt concentration. So, this method is ideal with the previous method also use a salt. So, you do not have to remove the salt as the step one. Then go other chromatography because some chromatography's will be hindered, if the salts are present, whereas HIC will work only on high salt concentration.

So, this is a very good technique and HIC ionic change chromatography, gel filtration chromatography. That is GPC they are useful to remove protein without effecting is activity. So, when I use HIC I am not affecting the activity of the protein, if I use ion exchange chromatography, I will not be affecting the activity of protein.

If I again use gel filtration or gel presentation chromatography. I am not affecting the activity of the protein, whereas some techniques may affect the activity of my protein. So, HIC is ideal for such situations.

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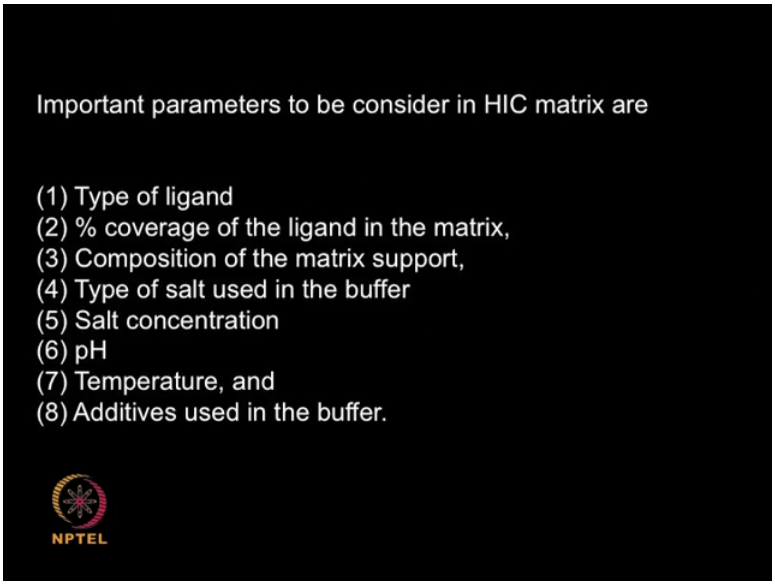

So, what are the important parameters we need to consider, when we are using HIC type of suppression type of ligand. What type of hydrophobic groups, am I using on top of my stationary matrix, what is the percentage coverage of the ligand on the matrix. Composition of the matrix support, what is the type of salt to used in the buffer, concentration of the salt, what is the pH, what is the temperature, what are the additives used in the buffer.

Are you using any, any other chemicals to change, certain surface properties, or you using some chemicals to dielectric constant of the solvent and so on. So, these are the parameters, which are going to affect the performance of the HIC. So, we can play around these parameters so that I am able to get a good suppression in a mixture of proteins based on HIC.

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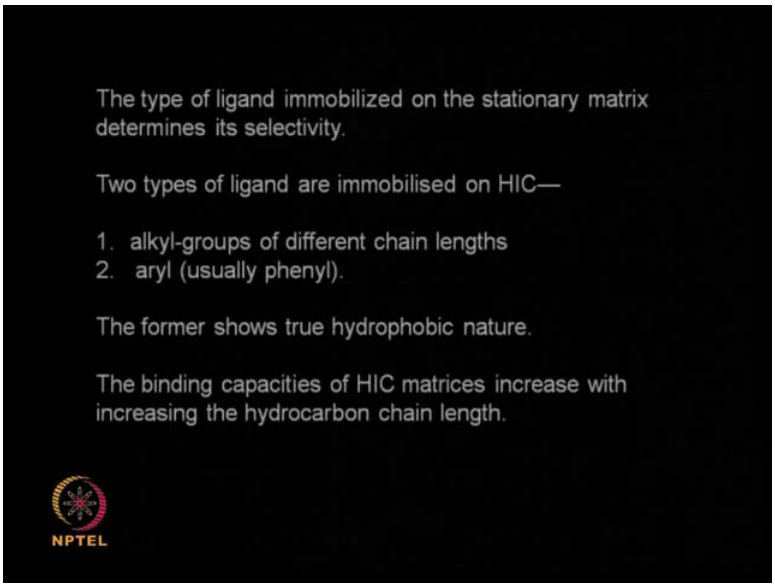

So, what are the types of ligands that are remobilized on the stationary phase because that determines the selectivity quit a lot. We can think about groups like alkyl groups of different chain lengths because alkyls are hydrophobic. So, we can have different chine lengths alkyl and their by increase the hydrophobic nature of the groups, or I can use aerial groups like final groups, they are also hydrophobic in nature.

So, I can have surfaces with the different alkyl chain length attached, which makes the surface hydrophobic in nature. So, if I have very long chine hydrocarbon then the binding capacity will be much higher, when compared to short chain hydrocarbons. So, the ligands that are immobilized are generally, hydrocarbon chain line or aryl type of groups.

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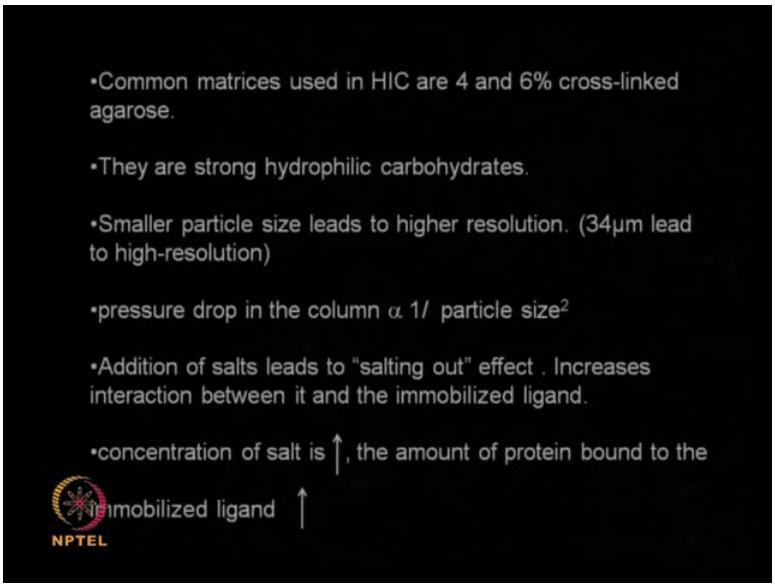

Common matrices used in hydrophobic interaction chromatography are about 4 to 6 percent cross linked agarose, but they are strong hydrophilic carbohydrates. Smaller particle size will need to higher resolution. So, like 34 micron particle will lead to very high resolution, but you need to consider that the pressure drop in the column will be inversely pronominal to the square of the particle size. That is pressure drop is proportional to 1 by particle size square. So, smaller the particle higher will be the pressure drop and its going to go up in terms of square.

So, if the particle size goes down, pressure dropping increases the pressure drop increases, the back pressure increases. That means, I need to have much more stronger pump, to pump the solution through the column. So, that is the big problem. So, we need to consider this aspect, and you cannot keep on reducing the particle size of the support. Addition of salt lead to salting out, we will talk about salting long time back. So, more salts you have in the solution, protein will start coming out and it will increase the interaction between the protein and the ligand.

So, the idea is to increase the interaction between the protein and ligand. So, what we do? We can add lot of salt with the concentration of salt is increased, the amount of protein bound to the remobilized ligand also increases its obvious, when I am adding more salt I am enhancing salting out. So, the proteins are going to precipitate out. Now, this proteins will interact more strongly with the ligand.

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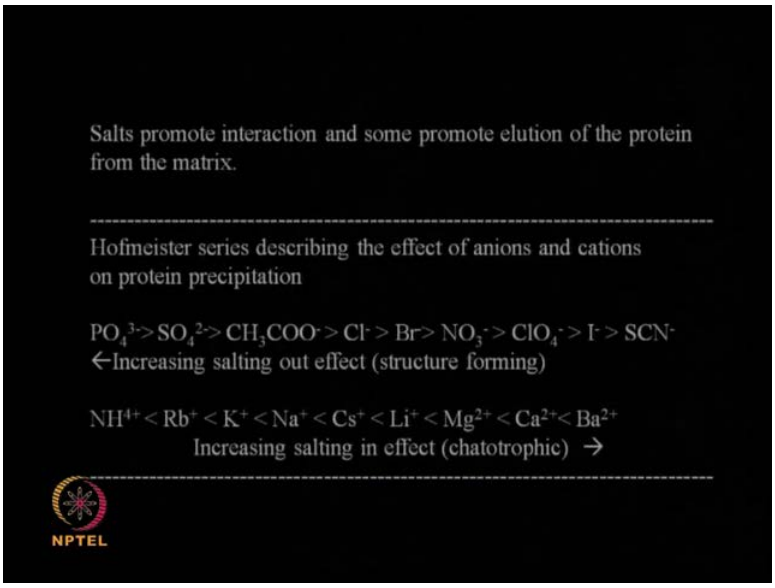

So, salt promotes interaction whereas, some salts promote elution of the protein from the matrix. That means, some salts can also help in the displacement of the protein bound to the matrix. So, there is something called Holfmeister series, which describes the effect of anions and cations on protein precipitation.

So, if you move from this end right and end, right going to the left hand end, we are increasing salting out effect this actually called structure forming. So, if I am moving from SCN minus to I minus ClO 4 minus N O 3 minus B r minus C l minus CH3 COO minus S O 4 2 minus P O 4 3 minus ion enhancing the salting out.

Whereas, conversely if I moving for this end to this end I am increasing this salting in effect that is called chatotrophic. So, if I moving from ammonium 4 plus to Rb plus K plus Na plus Cs plus L i plus M g 2 plus C a 2 plus barium 2 plus, I am increasing in a salting in effect.

That means, protein will dissolve more inside the solution. So, if I am moving from here to here that is from the right, and the left I am enhancing the solubility of the protein, or sorry I am enhancing the salting out effect. That is protein will be coming out where as, if I am moving in from this left hand end to right hand end I am enhancing the salting in effect.

So, this type of movement will enhance the interaction between the protein and the immobilized material. Whereas, when I am moving from this end to this end, I am increasing the solubility of the protein inside the solvent. So, I am decreasing the interaction between protein and the immobilized hydrophobic groups.

So, you can play around with the type of anions with the type of cation either to enhance, the interaction between the protein and the ligand, which is immobilized through hydrophobic interaction or I can reduce the interaction between the protein and the ligand.

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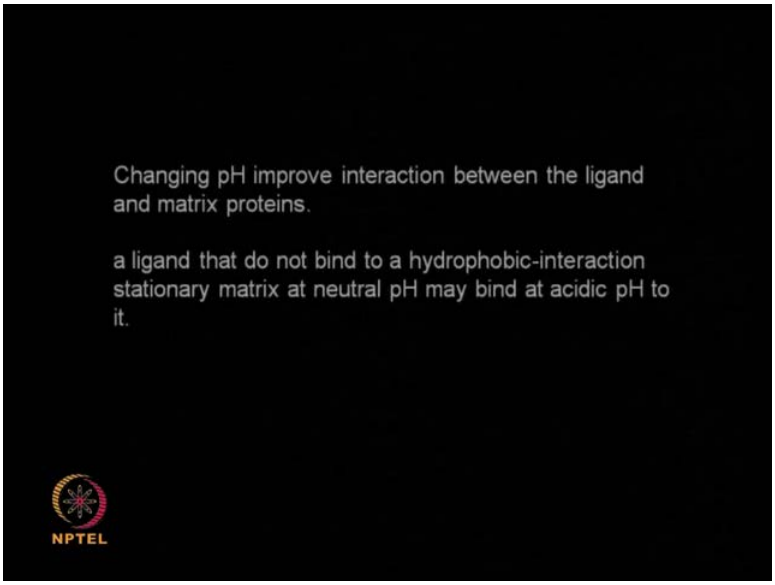

So, changing pH also improves between ligand matrix. So, we can play around with pH so, that we can enhance the interaction. For example, a ligand that do not bind to hydrophobic nitration stationary phase at neutral pH, may bind at acidic pH to it. So, I can make it slightly more acidic. So, that I can make the, the protein more bind to the stationary phase. Whereas, it might not be binding at the neutral pH.

Once, you have bound this protein next step will be regenerating the column and recovering this bound protein, right? That is called desorption, recovery, connection of whatever has bound to the matrix.

So, what do we do we decrease the concentration of salt. So, when we decrease the concentration of salt, what happen? That is something called salting in that may be

happening. So, the protein bound to the hydrophobic matrix will go into solution in the solution in the solvent originally, we had very concentration of salt. So, we had a salting out like effect now, will have lower concentration of salt. So, will we having something called a salting in type of effect.

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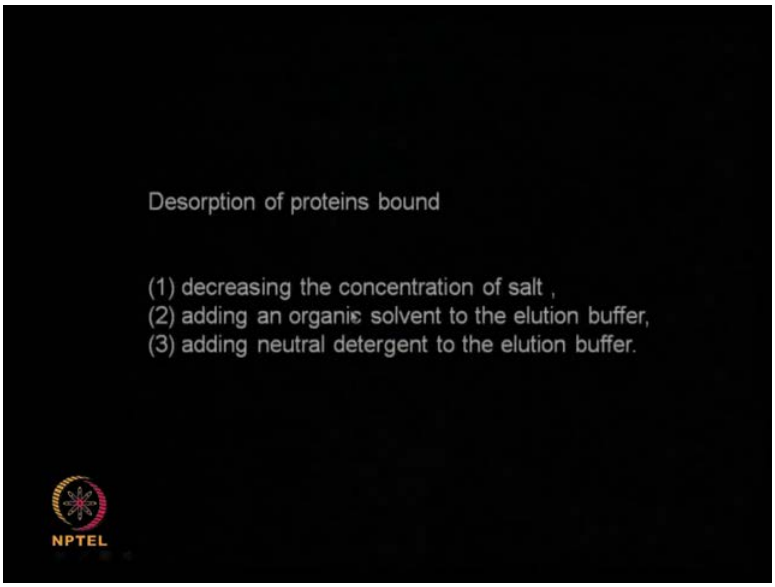

We can add an organic solvent. So, what are you doing? We are changing dielectric constant of the elution buffer. So, we can add an organic solvent, we are changing a dielectric constant. So, the proteins that's bound will get unbound, we can also add neutral detergent. That means, detergent is nothing but surfactant. So, I doing this we are try to solubilize the protein that is bound to the surface. So, these are some technique by which we can recover the protein that is bound to the immobilize surface, when we perform in hydrophobic interaction chromatography.

So, at the same time regenerating column for further studies. So, different ways one is changing the concentration of salts, second is by adding an organic solvent changing the dielectric constant, or adding some detergent neutral detergent. You do not want to add a cationic, or anionic type of detergent because that again going to contribute in the form of changing the concentration, but we want to add a neutral detergent.

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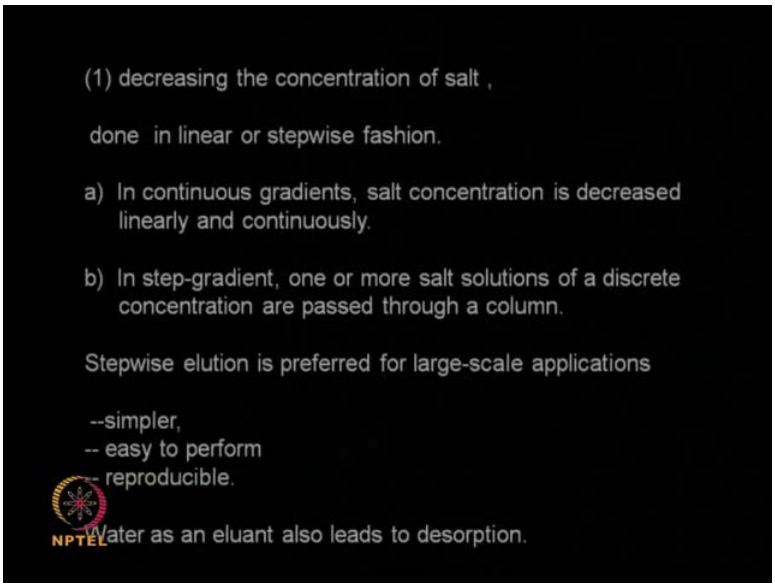

So, decreasing the concentration of the salt we can do we can a linear, step wise manner. So, we have a very high concentration of salt. So, we can slowly decrease in a very linear fashion over a period of time, or we can decrease it steps 2, 3 steps the concentration is broad down. So, in a continuous gradient salt concentration is decrease linearly, or continuously. In step gradient one or more salt solution of discrete concentration are pass through the column.

Step wise elution is preferred for large scale operation because doing a very uniform reduction in salt concentration over a period of long time, might not be possible. Whereas, we can go down in steps which is much simpler easy to perform, we can even reproduce it after 1 hour I reduce the salt from some value to half the value, then another after 1 hour I may reducing the salt concentration from that to lower value on so on. We can do this type of strategy for the recovery of the bound protein. Water as an eluant also has been tested that also, helps in these options of the proteins that is bound.

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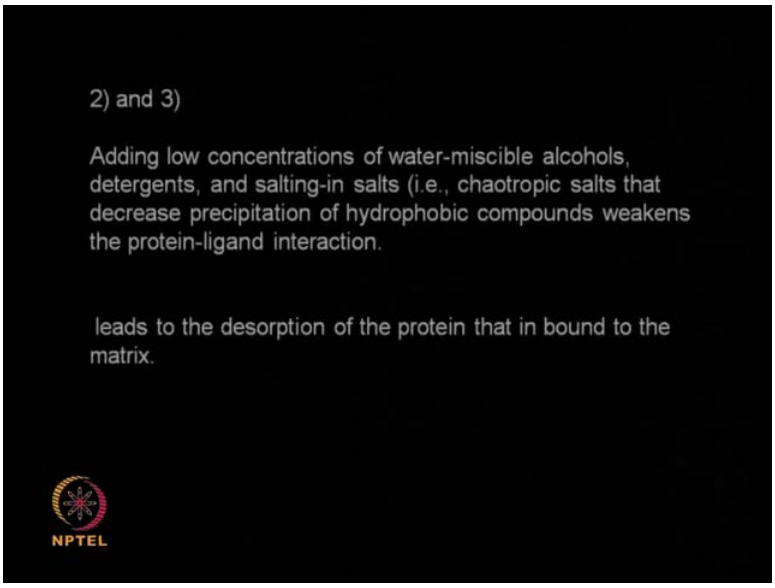

So, adding low concentrations of water miscible alcohols, detergents, salts like chatotropic salts that decrease precipitation of hydrophobic compounds weakens the protein ligand interaction. So, this is another way. First way method we looked at is changing in the salt concentration, but here we are talking about adding some solvents like alcohols, detergents that is surfactants or even salting in salts and so on. Now, all these when we do you are deserved in the protein that is bound to the matrix. So, this, these techniques help you to regenerate column, as well as recover has been bound to the column.

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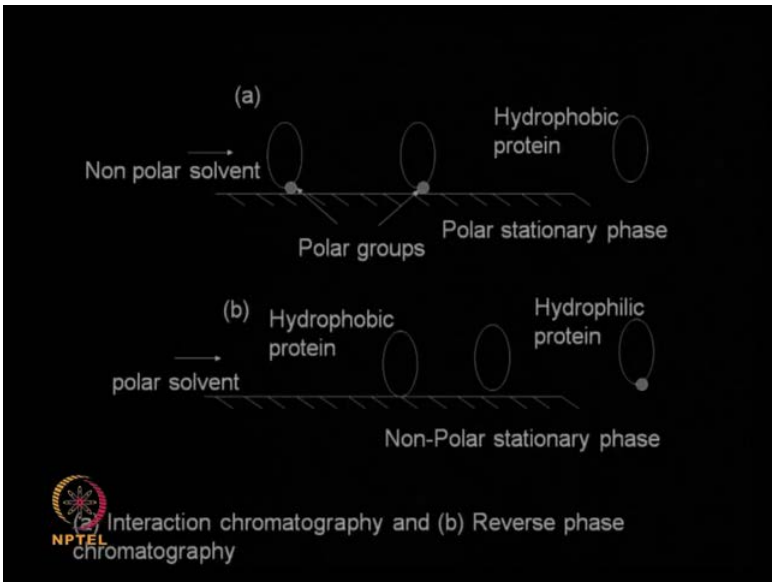

Now, if you look at a normal interaction chromatography. So, you have a polar stationary phase. So, you have your protein there are polar groups here. So, there is a interaction groups between polar group of the protein and the polar stationary phase. So, you will be using a non polar solvent here. So, hydrophobic proteins will come out first because there is no interaction between the hydrophobic proteins in the polar stationary phase. For example, silica or something and the polar proteins because they are going to interact with the polar stationary phase will get retarder, and they will come in the end.

Whereas, if you have non polar stationary phase like hydrophobic stationary phases and use a polar solvent, what will happen? Hydrophobic proteins will interact more with the non polar stationary phase. Whereas, hydrophilic proteins will not interact there will be coming out first, and the hydrophobic proteins will be retarded there will get slow down, there will come slowly later. So, in a interaction chromatography where you have a polar surface and a non polar solvent. The hydrophobic proteins will come out first, the polar groups or the polar proteins will come out later.

Whereas, if you take a hydrophobic type of interaction system or reverse phase chromatography will be having a non polar stationary phase, will be using a solvent. So, the hydrophilic proteins will come out first because it is not interact with the stationary phase. Whereas, the hydrophobic proteins will come out in the end because there will be interacting with the stationary phase.

So, two types of approaches we can use polar stationary phase and a non polar solvent, or we can use an non polar stationary phase and a polar solvent. So, both can be used in depending upon what you want to recover, what you want to separate, what does the mixture contain, what is the concentration of the protein of you interest, cost factors, time factors so on actually.

So, this hydrophobic system can be also called reverse phase chromatography because in a normal phase, we use a polar stationary phase. Whereas, in a reverse phase we are using a non polar stationary phase like a hydrocarbon, like a aryl group, long chain hydrocarbon. So, even in high performance liquid chromatography, we use hydrophobic stationary phase or reverse phase.

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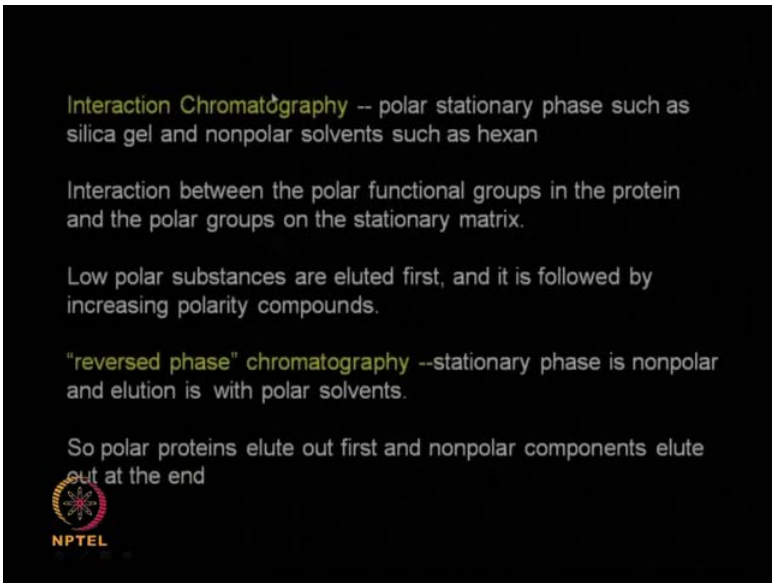

So, this interaction chromatography, we have a polar stationary phase such as silica gel, and non polar solvent such as hexane. So, there is an interaction between the polar functional groups in the protein and the polar groups in the stationary phase like N H, O H and so on. So, low polar substances are eluted first and then followed by increasing polarity. So, whereas, in the reverse phase or hydrophobic system the stationary phase is a non polar and the elution done to a polar solvent. So, polar solvents will elute out first non polar components will elute out at the end.

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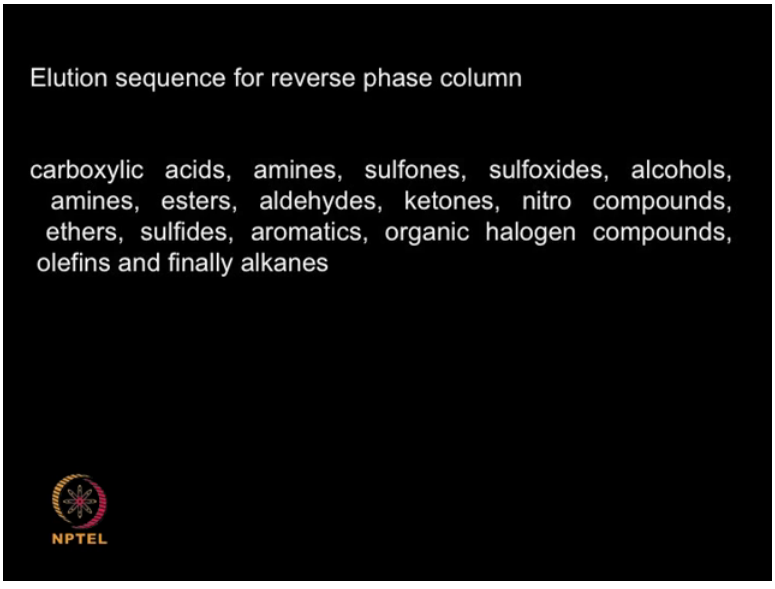

So, the elution sequence for the reverse phase will be carboxylic acids, amines, sulfones sulfoxides, algohols, amines, esters, aldehydes, ketones, nitro compounds, ethers, sulfides, aromatics, organic halogen compounds, olefins and finally, alkanes. So, for a reverse phase alkenes will come out in the ends because there is going to be interaction of alkane with the non polar functional groups present on the stationary phase. Whereas, the carboxylic acid, which are polar in nature will not interact and there will come out very fast.

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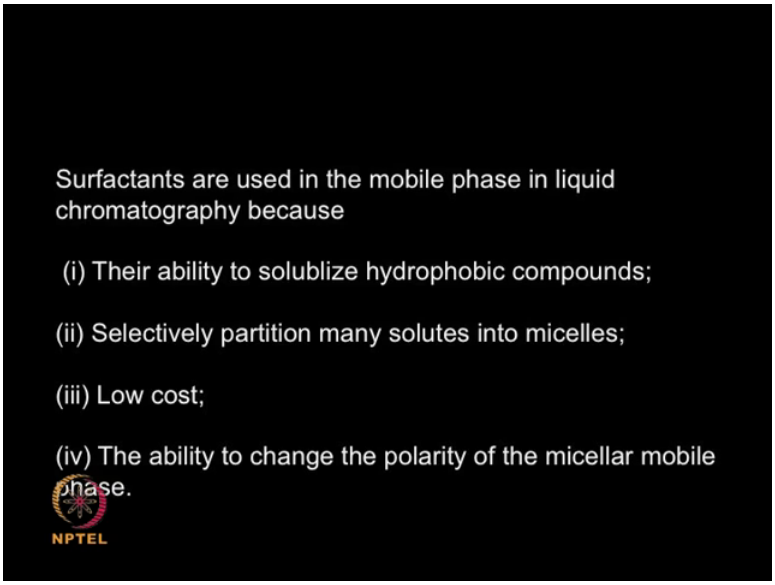

Sometimes surfactants are also used in the mobile phase, in liquid chromatography because they are very good in solublizing hydrophobic compounds. They can help in partitioning in many solutes into micelles, they are very low cost, they can also change the polarity of the mobile phase depending upon the type of surfactant we use. So, that is why surfactant are also used in liquid chromatography system, and they have good advantage.

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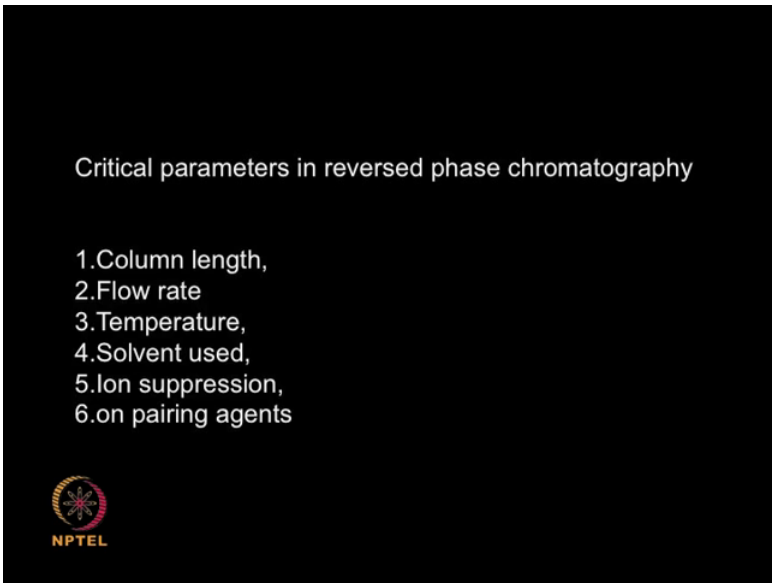

So, what are the critical parameter we need to consider in reverse phase. The column length how long you want to have the column. Flow rate flow rate of your continuous phase, what is the temperature you are working at, what are the solvents you are using because I said you generally, require a polar solvent because you are using a non polar or hydrophobic stationary phase.

Ion suppression how are you preventing, the suppression of ionization because once you have ionization, then the hydrophobic interactions will not take place. Ionizations spoils this type of hydrophobic interaction. So, what we do we need to change the pH so that ion suppression taking place.

That means, there is no dissociation of the component into ionizable specious. Use of ion tarring agent. So, all these parameters can help us to manipulate the process, or the efficiency or the selectivity of the reverse phase chromatography.

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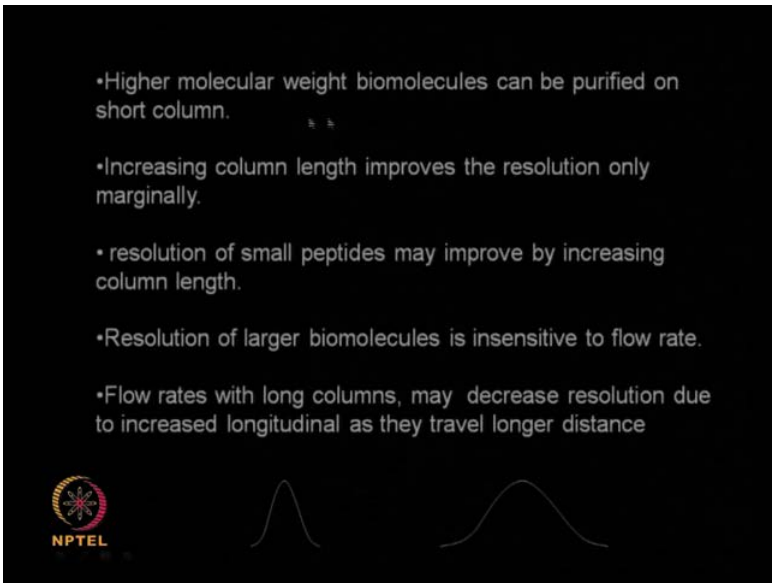

Higher molecular weight bio-molecules can be purified on short column, increasing column length improves the resolution only marginally. Whereas, if you have small peptides improve increasing column length improves the resolution. Resolution of large bio-molecules is insensitive to flow rate. The flow rates with the long columns may decrease resolution due to increased longitudinal distance. So, they have to travel longer distance in a long column so, that resolution gets decreased.

So, in a hydrophobic or the reverse phase system, the higher molecular bio-molecules can be purified in short column, increasing the column length is not going to improve your resolution, whereas if it is small peptides yes, we can improve the suppression with the longer columns. Resolution of larger bio-molecules is also insensitive to flow rates, and flow rates with long columns will decrees resolution due to increase longitudinal distance as the travel longer distance.

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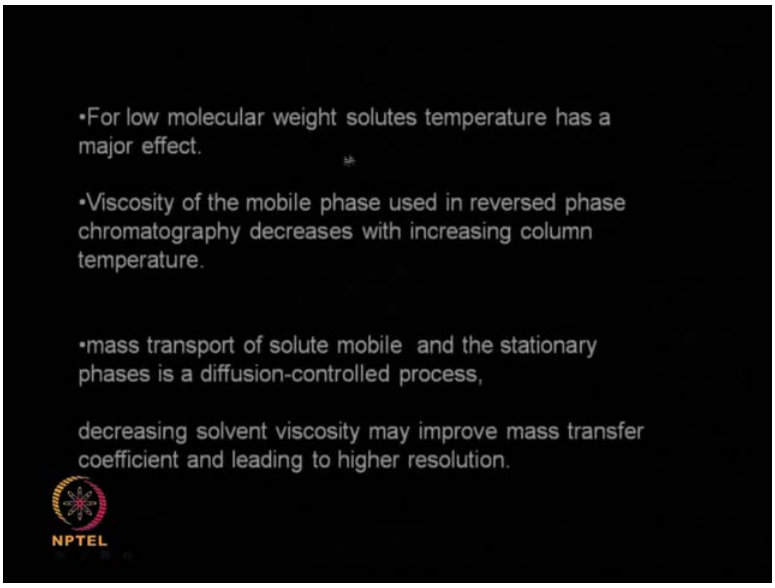

So, for low molecular weight solutes temperature has a bigger effect because if I increase column temperature I am affecting a viscosity of the mobile phase. When I effect the viscosity of the mobile phase, I am affecting the diffusions, I am effecting the dispersion, I am effecting the flow patterns. So, that will have a bigger effect because mass transport of solute from the mobile to the stationary phases is a diffusion controlled process. So, when I effecting the viscosity, it is going to effects by diffusion coefficient. So, decreasing solving viscosity will improve must and so, co-efficient leading to a resolution or higher resolution.

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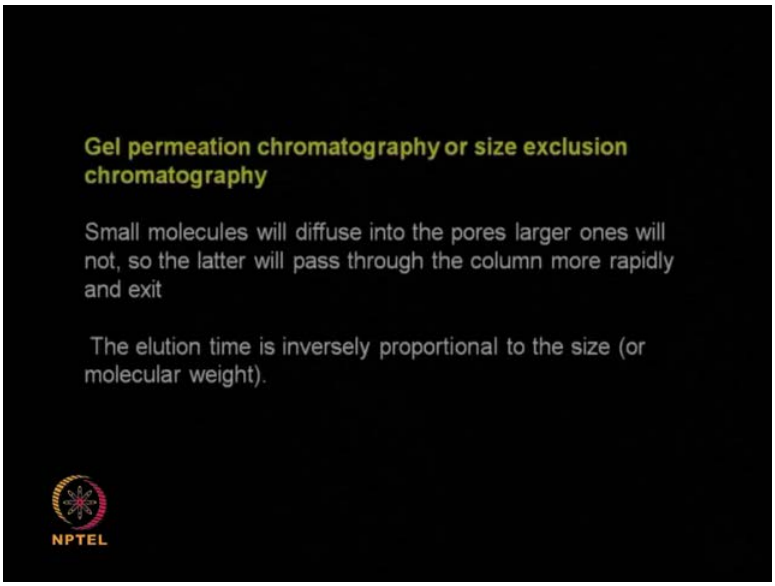

Now, we talked about hydrophobic interaction chromatography, we also mentioned about the concept of reverse phase chromatography. Originally, before that we talked about ion chromatography. Now, let us move to the next type of chromatography. Now, this is called gel permeation chromatography, or size exclusion chromatography. So, it is based on size, or it is based on molecular weight, assuming higher molecular weight are bigger is size.

For protein is higher molecular weight are bigger in size, smaller molecular weight are smaller in size. So, what you do here we have with stationary phase with pores so small molecules, which are smaller than the pore will defuse inside the pores get entrap, and get slow down. Whereas, large molecules which cannot enter the pores will keep travelling. So, they will rapidly come out on the other side they will exit faster.

So, large molecules will come out faster, small molecules will take much longer time to come out. So, the elution time is inversely proportional to the size of the molecules, or the molecular weight of the molecules. So, it is a very good technique to separate molecules of different size, or molecules of different molecular weight. It is called size exclusion chromatography or gel permeation chromatography.

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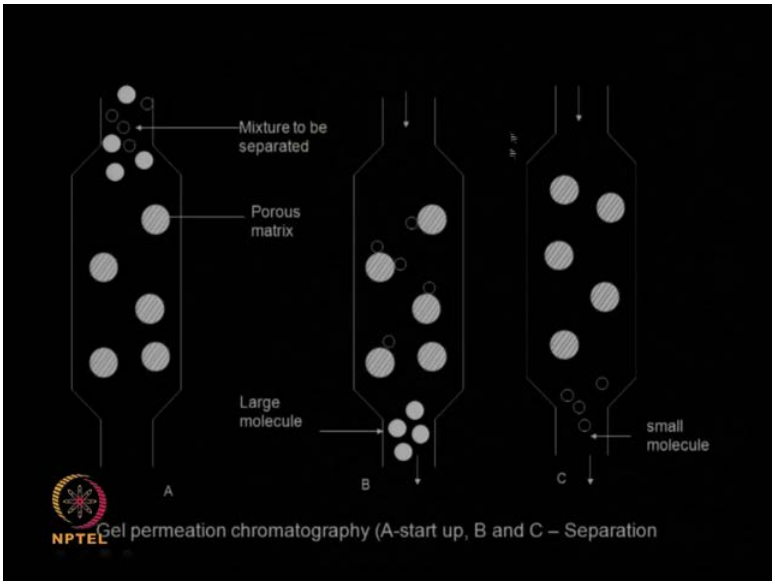

So, what happens we have pores matrix inside, we have big proteins, small proteins or proteins with higher molecular weight or proteins with smaller molecular weight. So, as the travel the smaller proteins get entrapped in that pores material. So, larger protein will

just come out, and after some time all the larger proteins have been removed and the small proteins will come later. So, elution time is proportional to the molecular weight or size of the proteins.

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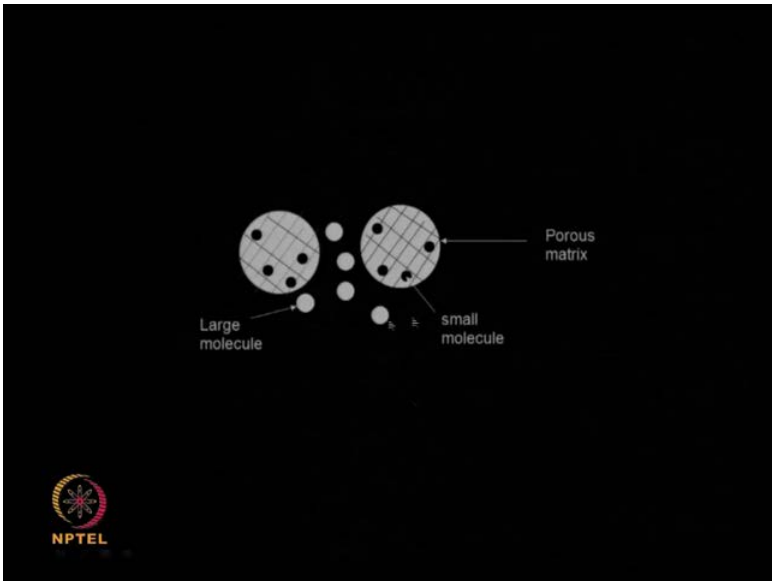

So, the small molecules get entrap in the pores larger molecules cannot enter the pores they. So, they travel faster that the principle of gel permeation chromatography, or molecular c-type of chromatography.

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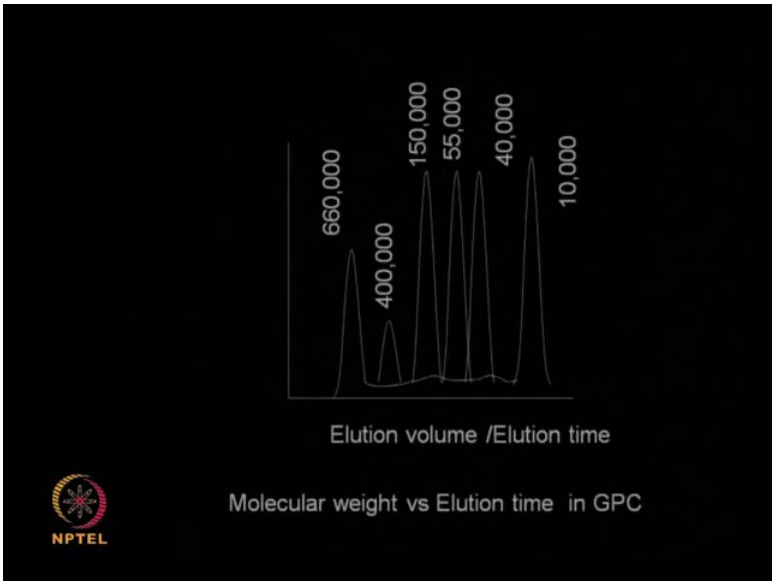

So, typically if you are looking at a elution volume or elution time you may have 660,000 molecular weight material coming out first followed by 400,000 molecular weight material followed by 150,000 molecular weight followed by material 55,000 molecular material, 40,000 finally, 10,000. So, you can see that molecular weight from 660,000 to 10,000 could be nicely separated out using this gel permeation chromatography, or size exclusion chromatography.

So, it is extremely useful for separating out useful, we can even measure the molecular weight of unknown protein if we know molecular weight of certain proteins. So, we can use it as a analytical tool, we can use it as a separating toll as well. So, it can be used both in analytical cemetery as well as in purification area.

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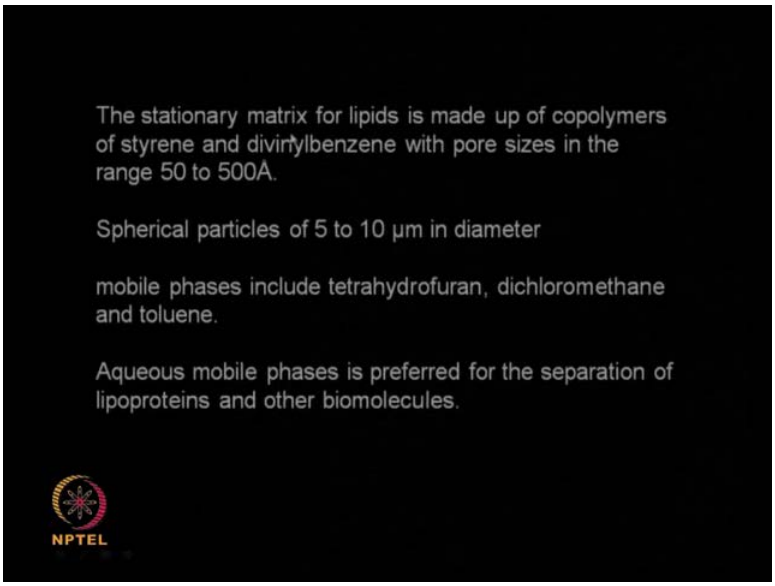

So, the stationary matrix for lipid is made up of co-polymers of styrene and divinylibenzence. So, it will have pore size in the range 50 to 500 Armstrong. So, it will be vertical particles 5 to 10 micron in diameter, the mobile phase can be tetra hydrofuran, dicloromethene toluene. So, if you are talking about lipoproteins, bio-molecules and so on we can even use aqueous mobile phase. So, these gel permeation chromatography can be used for proteins bio-molecules, it can used for small molecules, it can be used for synthetic polymers natural polymers and so on.

As I said it can used for both purification as well as analysis. Suppose, I have a protein of unknown molecular weight and if you have few proteins of known molecular weight, I

can get a standard graph the elution time of the known proteins. These are which there molecular weight and if I get the elution time for the unknown protein form the standard original graph, I can calculate what will be the molecular weight of the unknown protein. So, it is quite a power full technique in the area of analytical chemistry, and it can also be used for purification based on the size, or purification weight on the molecular weight.

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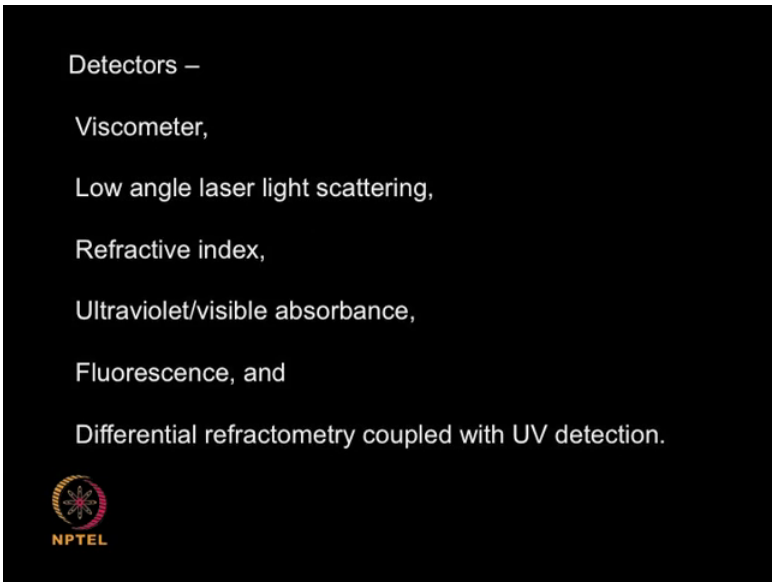

What are the types of detectors we can use? We can use viscometer type of detector, we can use low angle laser light scattering detector, we can use refractive index detector R I detector, we can use U V detector, visible range, we can use fluorescence detector, we can use differential refractometry coupled with U V detection. So, large number of detector can be used depending upon the characteristics of the protein, or metabolites or small molecule or synthetic polymer or natural polymer you are trying to separate out.

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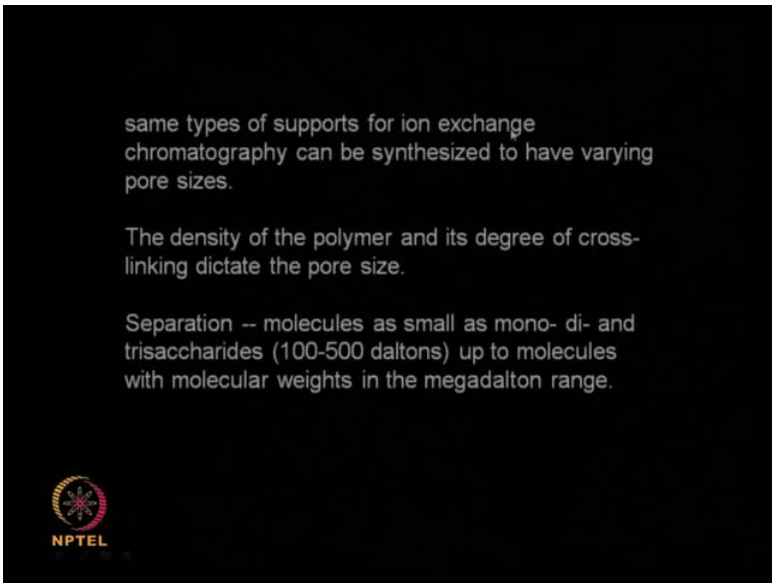

So, this supports or also similar to the supports that are used in ion exchange chromatography, and we can synthesis them the ring pore size because this technique is totally based on pore size. Smaller pores will allow small molecules to get entrapped, larger will allow only large molecules to get entrapped. So, if I have small pores larger molecules will not be able to entire. So, it is based on the concept of size. So, we can synthesis pores of different sizes.

So, the pore size is also a function of density of the polymer, and also the degree of the cross linking. So, I can manipulated the degree of cross linking, I can manipulate the density and achieve and different pore sizes. So, we can even separate molecules as small as mono di trisaccharides. That is 100 to 500 daltons also can separated, going right up to mega daltons a at 10 power 6, 10 power 7 dalton molecular weight.

So, this technique is very power full for separating out from a very small range, in terms of 500 daltons going up to million or 10 million daltons as well. So, we will continue a next topic of chromatography in our next class.